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This study tests whether breast cancer can be eliminated by immunization with foreign peptides followed by delivery of peptides to tumors. We proposed to (1) establish an in vitro assay to measure tumor growth inhibition, (2) synthesize pro-peptides for activation at the tumor site by beta-glucuronidase, and (3) test tumor rejection with peptide therapy. The 3-D tumor growth inhibition assay is established. D2F2 mammary tumor and another tumor line 168 with metastatic potential were inhibited by CTL in the 3-D matrix. HLA-A2.1 associated Flu peptide MP-58 GILGFVFTL was used for pro-peptide development. Pro-peptide was synthesized with a minimal protection approach. Purification of the pro-peptide to high purity is the current goal. To test therapeutic effect, mice were immunized with MHC I p876 and MHC II PADRE, injected with peptide loaded tumor cells, followed by local peptide injections. Complete tumor rejection was achieved. Challenging tumor cells not pre-coated with peptide were not rejected by p876 treatment. Vaccination and therapy with PADRE alone demonstrated anti-tumor effect and this activity is being further pursued. If MHC I peptide can be delivered directly unto tumor cells in the form of pro-peptide, combined treatment with PADRE may exert enhanced efficacy.

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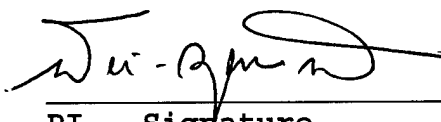
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INTRODUCTION

The goal of this project is to test a new concept in anti-cancer immunotherapy using pro-peptides as the therapeutic agents. The proposed tasks are the following.

- Task 1 Establish an in vitro assay to measure tumor growth inhibition by peptide specific T cells and pro-peptides
- Task 2 Synthesize and test the activity of pro-peptides to be activated by β -glucuronidase at the tumor site
 - (A) Synthesis of glucuronide derivatives of β -gal peptide TPHPARIGL.
 - (B) Measurement of peptide and pro-peptide binding to MHC.
- Task 3 Test tumor rejection in mice immunized with the foreign peptides and treated with the pro-peptides.
 - (A) Measurement of β -glucuronidase activity in mouse mammary tumors.
 - (B) Measurement of reactive T cell frequency in peptide immunized mice.
 - (C) Measurement of pro-peptide and peptide distribution in vivo.
 - (D) Measurement of tumor growth inhibition in mice.

BODY

Task 1 Establish an in vitro assay to measure tumor growth inhibition by peptide specific T cells and pro-peptides
(Results from this study have been submitted for publication, Wei WZ et.al. Foreign peptides delivered to the tumor sites are effective anti-cancer agents, pending revision.)

A three dimensional tumor growth inhibition assay has been established using rat tail collagen and was used to test the activity of CTL specific for β -gal peptide p876 TPHPARIGL. D2F2 tumor cells and CTL were embedded together in 1 μ l of collagen (**Figure 1A**) and incubated overnight. When the cell bolus was established, p876 at 5 or 20 μ g/ml was added to the culture. D2F2 cells co-embedded with anti-p876 CTL, but without exogenous peptide grew progressively. The addition of p876 resulted in complete inhibition of tumor growth, supporting anti-tumor activity of p876 specific CTL.

To test whether CTL can migrate through the matrix to destroy the target tumor, D2F2 mammary tumor cells were embedded in the collagen, incubated overnight and CTL were added on top of the gel. Approximately 20-40% of CTL migrated through the collagen in 18 hrs and floating T cells were removed (1). Peptide p876 at 5 or 20 μ g/ml was added and complete disintegration of the tumor boluses was observed after 5 days of culture. This assay system was used to test destruction of an independent mouse mammary tumor line 168 by migratory anti-p876 CTL which has demonstrated metastatic activity (**Figure 1B**). Destruction of the cell bolus was observed, supporting anti-tumor activity of anti-p876 CTL and the validity of the 3-D tumor growth inhibition system.

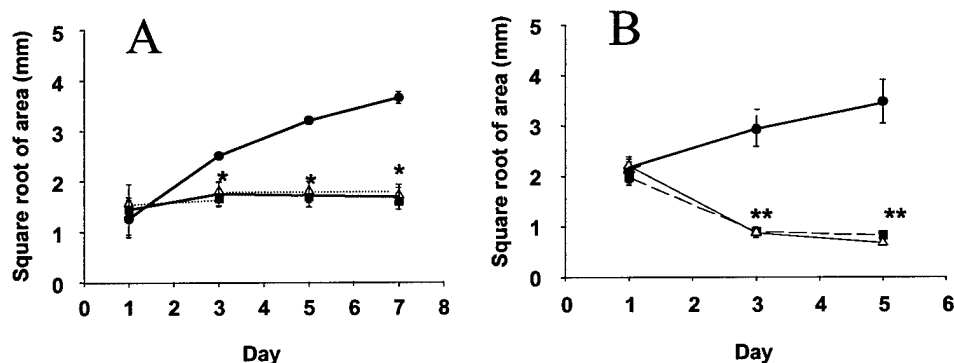
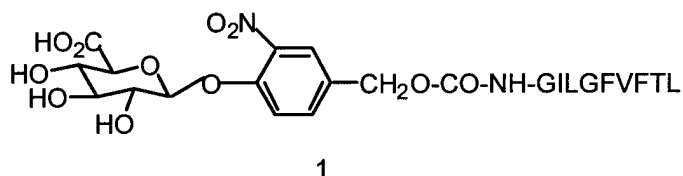


Figure 1 Inhibition of D2F2 tumor growth by anti-p876 CTL in 3-D collagen gel. (A) A total of 1×10^5 D2F2 cells were co-embedded with 3×10^5 anti-P876 CTL in 1 μ l of collagen. After overnight incubation, p876 at 5 (solid square) or 20 (open triangle) μ g/ml was added to the culture. Control group (solid circle) did not receive peptide. (B) 1×10^5 BALB/c 168 tumor cells were embedded alone in 1 μ l of collagen and 3×10^5 anti-P876 CTL were added to the collagen gel after a tumor mass is established overnight. CTL migrated through the gel and P876 peptide was added after 24 hrs. Tumor growth was measured by the square root of the growth area. * indicates significant difference between the control and test groups at $p < 0.005$.

Task 2 Synthesize and test the activity of pro-peptides to be activated by β -glucuronidase at the tumor site

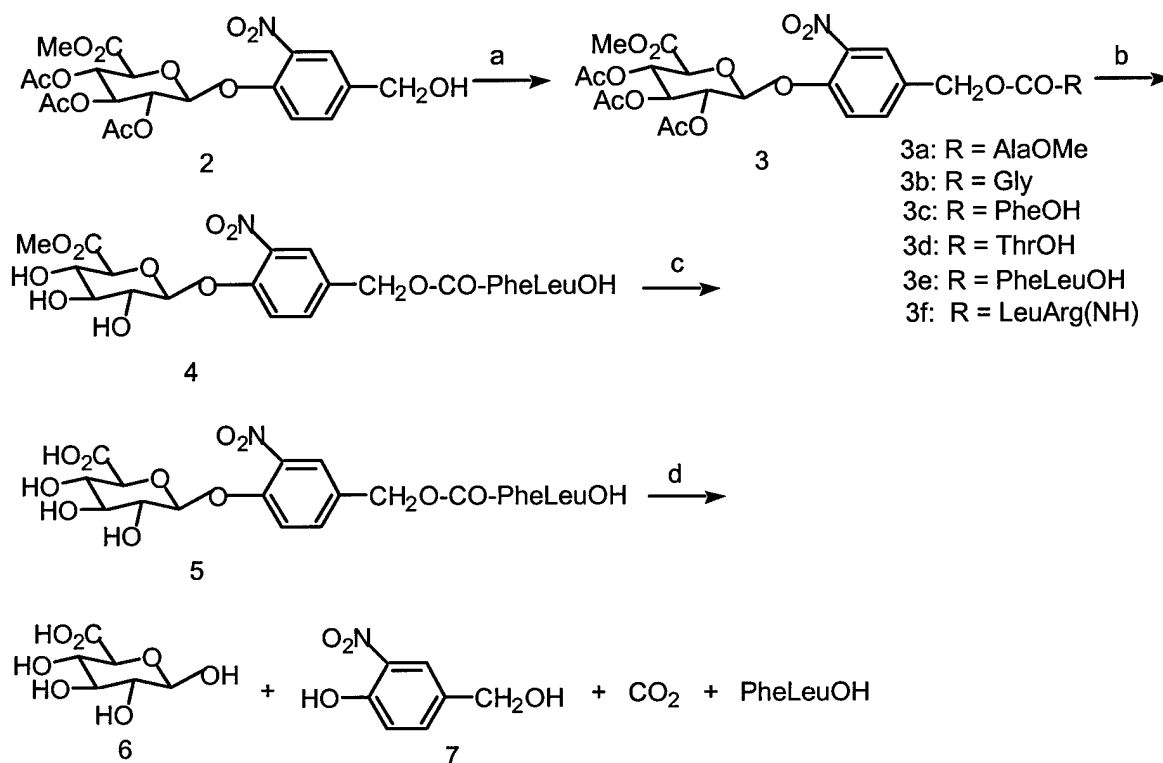
(A) Synthesis of glucuronide derivatives of β -gal peptide TPHPARIGL.

Flu peptide MP-58 GILGFVFTL presented by human HLA-A2.1 rather than beta-galactosidase peptide TPHPARIGL presented by K^d was used for pro-peptide development because the residues are less reactive and there is potential human application. In vivo activity of GILGFVFTL pro-peptide can be tested in A2.1/ K^b transgenic mice maintained in our animal care facility. One of the major goals of this project is elaboration of synthetic methods for therapeutic pro-peptides capable of delivering antigenic peptides to the tumor cells. Our approach is based on a similar strategy used for the synthesis of β -glucuronide attached through a self-immolative linker to antitumor antibiotic such as doxorubicin. Our immediate target is β -glucuronide of 4-hydroxy-2-nitro-phenylmethoxycarbonyl derivative of immunogenic peptide GILGFVFTL (formula 1).



Model experiments have indicated that this approach is applicable for amino acids and peptides (Scheme 1). Thus, methyl tri-O-acetyl- β -D-glucuronide of 4-hydroxy-2-nitrophenylmethanol (2) was reacted after activation with N,N'-disuccinylimidyl carbonate (DSC) with the following model compounds: AlaOMe, GlyOH, PheOH, ThrOH, PheLeuOH and LeuArgOH to give protected compounds 3a - 3f. In all instances except LeuArgOH, the conjugation took place at the N-terminal amino group in 56 - 73 % yield. With basic dipeptide LeuArgOH, the reaction occurred at the guanidino function of Arg (41 % yield).

Scheme 1



a. 1. NEt₃, N,N'-Disuccinyliminocarbonate (DSC), MeCN.

2. R-H (for R see 3a - 3f), NEt₃, DMF.

b. MeONa, MeOH.

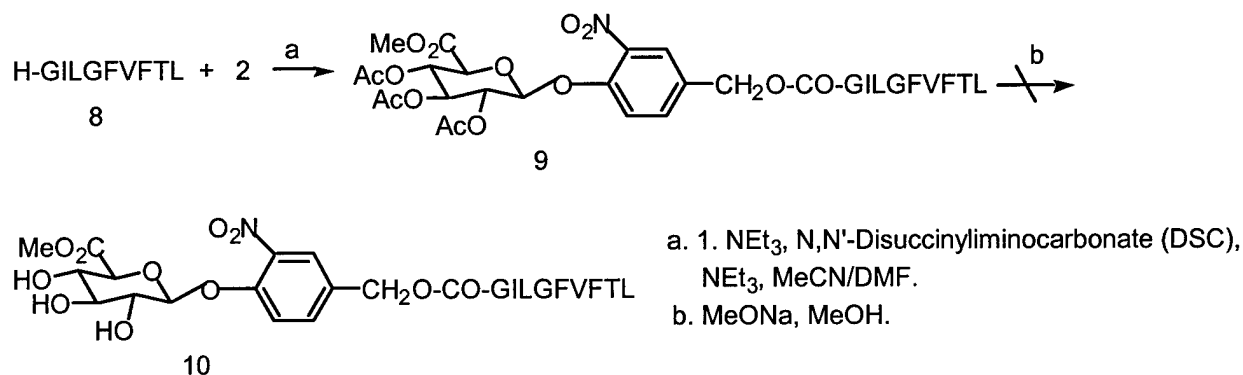
d. PLE, pH 7.2.

e. β -Glucuronidase, pH 7.2.

The dipeptide derivative 3e was deacetylated with MeONa in MeOH to give glucuronide 4 (76 %). The carbomethoxy group was then hydrolyzed using pig liver esterase (PLE) to give deprotected prodrug 5 in 91 % yield. Digestion of 5 with β -glucuronidase was also smooth to furnish alcohol 7 and starting dipeptide PheLeuOH detectable by HPLC. β -Glucuronic acid (6) could not be detected by HPLC (lack of UV absorption). These results reinforced our initial supposition that the designed approach for synthesis of peptide prodrugs is workable.

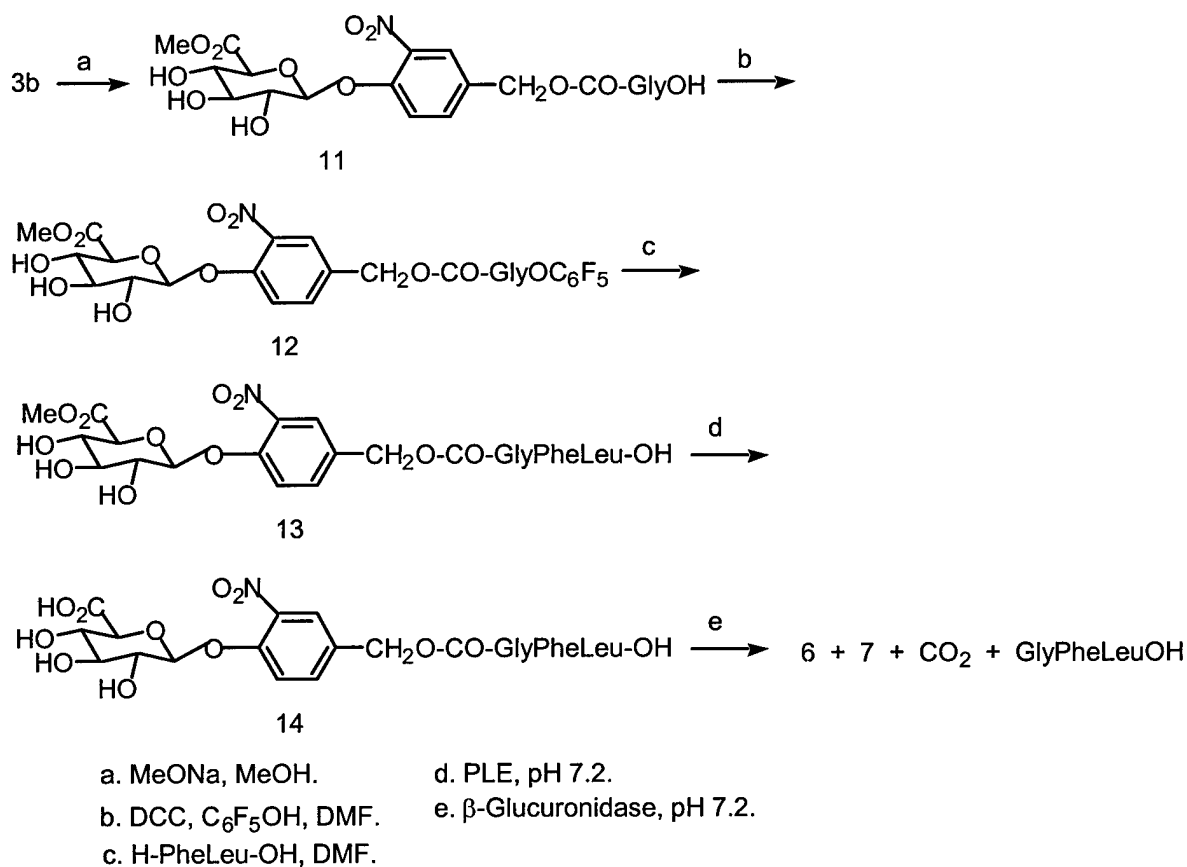
Commercial nonapeptide H-GILGFVFTL-OH (8) was then reacted with methyl tri-O-acetyl- β -glucuronide of 4-hydroxy-2-nitrophenylmethanol (2) activated with DSC to give the protected conjugate 9 (50 %, Scheme 2). The mass spectra (MS) showed a peak of m/z 1500 ($M + Na$) corresponding to the expected conjugate. However, MS of the product of deacetylation of 9 with MeONa in MeOH failed to show m/z 1350 which would indicate a presence of compound 10. In addition, HPLC showed that the product was a mixture of several different components. Lesser stability of oligopeptide toward a strong base (MeONa) may possibly be responsible for complexity of the product.

Scheme 2



Therefore, an alternative protocol was adopted (Scheme 3) (2). The protected glycine derivative 3b was deacetylated with MeONa in MeOH to give compound 11 (79 %). The latter product was converted to

Scheme 3



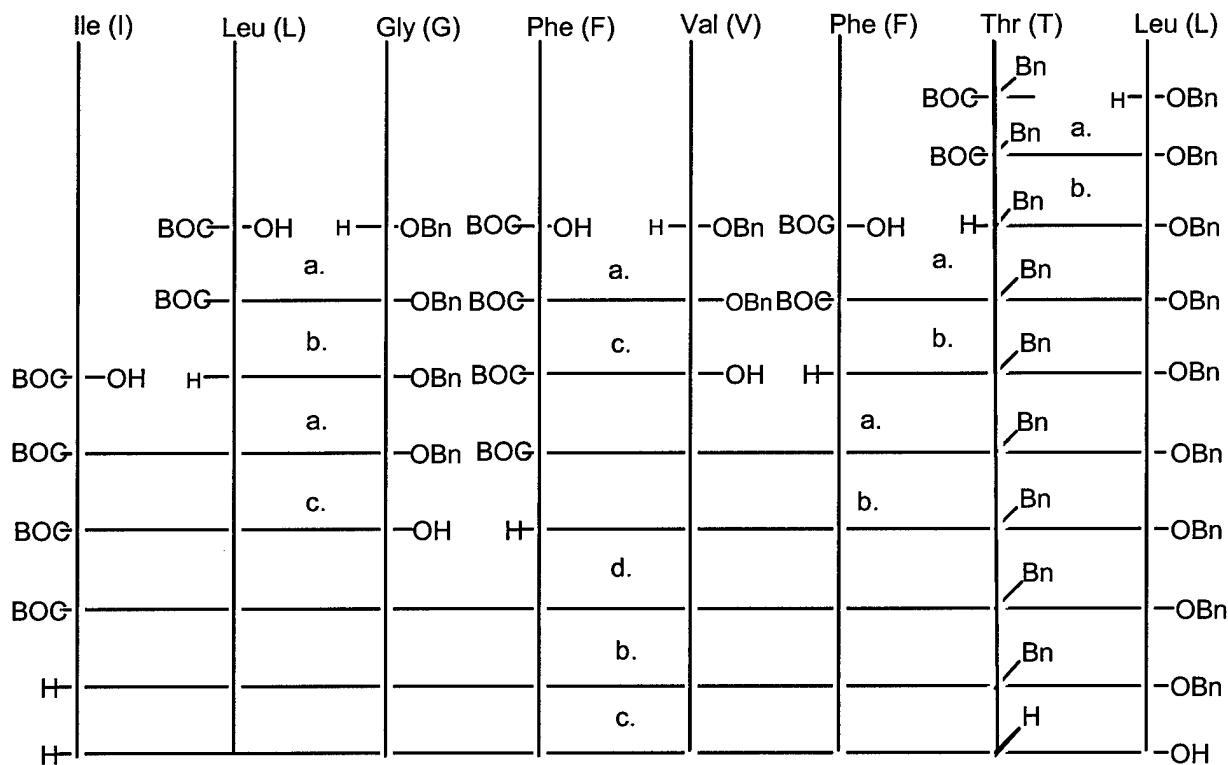
pentafluorophenyl ester 12 using dicyclohexylcarbodiimide (DCC) and pentafluorophenol in 89 % yield. Intermediate 12 was then treated with the dipeptide H-PheLeu-OH to obtain the tripeptide conjugate 13 (62 %). The carbomethoxy group was hydrolyzed using PLE to give the deprotected derivative 14 (92 %). Digestion of 14 with *E. coli* or mammalian β -glucuronidase furnished alcohol 7 and tripeptide

GlyPheLeuOH detected by HPLC. These results indicated that the hydroxyl groups of a sugar moiety do not interfere with formation of the conjugate and, thus, minimal protection strategy can be used.

The octapeptide H-ILGFVFTL-OH (15) /m/z 909 (M + H)/ was synthesized in solution using segment condensation strategy (Scheme 4). The parent immunoactive nonapeptide H-GILGFVFTL-OH /m/z 966, M + H/ was also synthesized for comparison from octapeptide 15 but its biological activity was about half of that of commercial sample. The HPLC and mass spectra have shown that both samples of nonapeptide contain a by-product which was more abundant in the peptide resultant from solution synthesis. The pentafluorophenyl ester derivative 12 was treated with the octapeptide H-ILGFVFTL-OH (15) to obtain conjugate 16 /m/z 1349 (M - H)/ in 61 % yield (Scheme 5). In contrast to tripeptide 13, conjugate 16 was not a substrate for PLE. Alternatively, carbomethoxy group of 16 was hydrolyzed using Ba(OH)₂ to give the deprotected prodrug 17 (70 %) of m/z 1335 (M-H). Digestion of 17 with mammalian β -glucuronidase gave alcohol 7 and nonapeptide 8 detected by HPLC. Apparently, the minimal protection approach is applicable for larger peptides. Obtaining materials 8, 9 and prodrug 15 in desirable purity for biological tests is currently in progress using preparative HPLC.

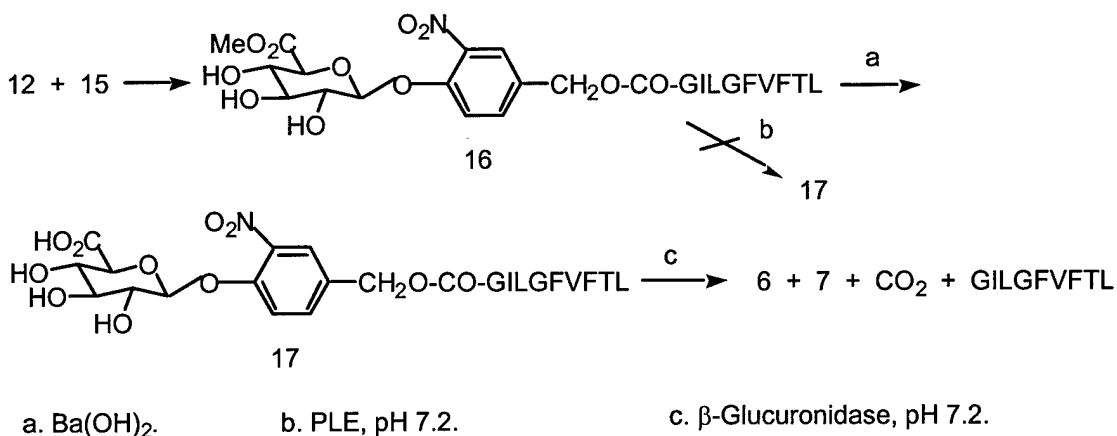
Scheme 4

Synthesis of peptide ILGFVFTL (15):



- a. DCC, HOBT, THF - DMF, 0°C. WSCD-HCl, water-soluble carbodiimide hydrochloride,
 b. HCO₂H. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
 c. H₂, Pd/C, EtOH.
 d. WSCD-HCl, HOBT, THF-DMF, 0°C.

Scheme 5



Task 3 Test tumor rejection in mice immunized with the foreign peptides and treated with the pro-peptides

(D) Measurement of tumor growth inhibition in mice.

(Results from this study have been submitted for publication, Wei WZ et.al. Foreign peptides delivered to the tumor sites are effective anti-cancer agents, pending revision.)

Effect of p876 on tumor growth in BALB/c mice

To test if anti-p876 CTL can inflict the same anti-tumor activity *in vivo*, BALB/c mice were immunized with *AdCMVLacZ* to activate β -gal specific CTL and boosted in two weeks with 100 μ g p876 in IFA. At two weeks after the second immunization, mice were challenged with D2F2 cells pre-coated with p876. Starting the day after tumor cell challenge, mice were injected at the tumor site, every other day, with 200 μ g of p876 in 0.1 ml saline. There were five injections in 10 days. Control mice received saline or were not treated. One group received uncoated D2F2 cells. There was no demonstrable protection in any of the treatment group (not shown). The inability to inhibit tumor growth by local administration of p876 may be due to (1) inadequate number of CTL at the tumor site, (2) inadequate cytokine or other signals to sustain CTL activity at the tumor site, or (3) destruction of normal rather than tumor cells marked by the injected peptide.

Therapeutic effect of p876 and PADRE

To enhance local recruitment or maintenance of CTL, CD4 T cell reactive peptide was incorporated into the treatment. PADRE which binds to a wide range of human and murine MHC II was tested as local CD4 T cell activator (3). BALB/c mice were immunized with *AdCMVLacZ* and boosted in 2 wks with 100 μ g p876 and 20 μ g PADRE in IFA. At two weeks after the second immunization, mice received s.c. D2F2 cells pre-loaded with p876 and PADRE. Mice were treated five times at the tumor site with a mixture of PADRE and p876, every other day. Complete inhibition of D2F2 tumor growth was observed. These results suggested that PADRE activated CD4 T cells at the tumor site to enhance p876 mediated CTL activity and resulted in tumor rejection.

We further tested the efficacy of treating D2F2 tumors which were not pre-loaded with exogenous peptides. Mice were immunized i.c. with AdCMVlacZ and boosted in two weeks with p876 and PADRE. Mice received untreated D2F2 cells two weeks later and peptide injection at the tumor site starting 2 days after tumor challenge. **Figure 2** shows tumor rejection in 5 of 8 mice treated with PADRE and 4 of 8 mice treated with a mixture of p876 and PADRE. P876 alone had no therapeutic effect. It remains to be determined if treatment with PADRE and MHC I pro-peptides which are designed for local release will result in enhanced tumor rejection. Local treatment with MHC II binding peptide alone appeared to inhibit the growth of D2F2 tumor cells which do not express class II MHC. This finding is further analyzed.

To test the therapeutic activity of PADRE alone, mice were immunized with 20 µg of PADRE in CFA and boosted with the same peptide in IFA. Injection of D2F2 tumor cells and PADRE peptide at the tumor site were performed as before. Again, half of PADRE treated mice rejected the tumor (**Figure 3**). Mice in the control group were mock immunized with CFA and IFA and injected five times with PADRE at the tumor site. There was no therapeutic effect with this treatment. Therefore, anti-tumor activity of locally delivered PADRE was mediated by previously immunized effector cells.

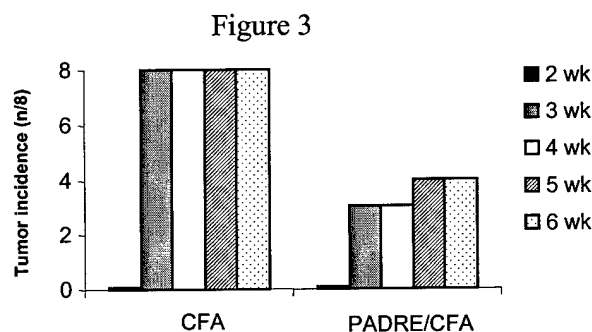
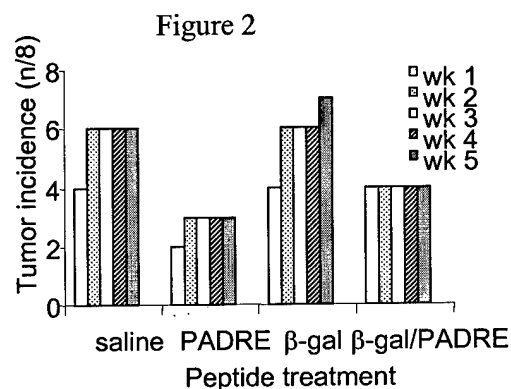


Figure 2 Therapeutic effect of PADRE and p876. BALB/c mice were immunized with AdCMVlacZ, PADRE and p876 as described in the legend of Figure 3. At two wks after vaccination, each mouse received s.c. 1×10^5 naïve D2F2. Mice were divided into four groups, each receiving Saline, 40 µg PADRE, 200 µg p876 and a combination of PADRE and p876 every other day for 10 days. The lower panel showed the average tumor diameter in test and control mice.

Figure 3 Therapeutic effect of PADRE. BALB/c mice were immunized with 20 µg of PADRE in CFA, boosted in two weeks with the same peptide in IFA. At two weeks after vaccination, each mouse received 1×10^5 naïve D2F2 and five PADRE treatment. Control group were immunized with CFA and IFA without PADRE and were treated with PADRE five times.

KEY RESEARCH ACCOMPLISHMENTS

1. Establish an *in vitro* 3-D tumor growth inhibition assay to measure anti-tumor activity of peptide specific cytotoxic T cells
2. Establish the conditions for the synthesis of glucuronide derivatives of Flu peptide MP-58 GILGFVFTL
3. Demonstrate tumor rejection in mice immunized to MHC I p876 and MHC II PADRE peptide and treated by local injection of both peptides. p876 must be pre-loaded on

tumor cells to achieve the therapeutic effect. In vivo loading of MHC I peptide on tumor cells may be achieved with pro-peptides which are designed for release at the tumor sites and this will be tested.

REPORTABLE OUTCOMES

Wei WZ, Ratner S, Shibuya T, Yoo G, and Jani A, Foreign peptides delivered to the tumor sites are effective anti-cancer agents. *Int. J. Cancer*, pending revision.

Wei, WZ., Shibuya, T., Yoo, G., Ratner, S., Foreign peptides as therapeutic agents against solid tumors, *Proc. AACR.*, 41: 114, 2000

CONCLUSIONS

In the 1 yr funding period, three major tasks have been initiated with significant progress. For Task 1, the in vitro tumor growth inhibition assay in the 3-dimensional collagen gel was established. Complete inhibition and disintegration of tumor cell boluses by peptide specific CTL was observed with two different mammary tumor lines. For Tasks 2, the conditions for pro-peptide synthesis has been established using the minimal protection approach, supporting the feasibility of the proposed synthesis task. Purification of the pro-peptide to desired purity will be the major effort. For task 3, the therapeutic effect of peptide therapy was demonstrated in mice immunized with the peptides, challenged with peptides loaded tumor cells and treated by local injection of peptides. Administration of MHC II associated PADRE was necessary for the therapeutic effect. Pre-loading of MHC I may be replaced with pro-peptide. Surprisingly, injection of PADRE alone has therapeutic effect. The results from Tasks 1 and 3 have been reported in the annual meeting of American Association of Cancer Research, 2000 and a manuscript has been submitted for publication. It continues to be our goal to develop effective anti-tumor therapeutic peptides and the results to date support the feasibility of this approach.

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2. Reimer *et al* *J. Chem.Soc. Perkin Trans 1*:925-932 (1993)
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APPENDICES

1. Wei, WZ., Shibuya, T., Yoo, G., Ratner, S., Foreign peptides as therapeutic agents against solid tumors, *Proc. AACR.*, 41: 114, 2000
2. Wei WZ, Ratner S, Shibuya T, Yoo G, and Jani A, Foreign peptides delivered to the tumor sites are effective anti-cancer agents. *Int. J. Cancer*, pending revision.

Appendix A

Proc. AACR., 41: 114, 2000

Foreign peptides as therapeutic agents against solid tumors

Wei-Zen Wei, Terry Shibuya, George Yoo and Stuart Ratner, Karmanos Cancer Institute, Detroit, MI 48201

CD4 and CD8 T cells are readily activated by immunogenic peptides, whether naturally occurring or artificially designed. These T cells are potent anti-tumor effectors if the corresponding peptides are present on the tumor or neighboring cells. Taking advantage of such effector functions, foreign peptides were injected into the tumor sites in peptide immunized mice and tested as anti-cancer therapeutics. The test agents were beta-galactosidase (β -gal) peptide p876 associated with L^d and Pan DR Reactive Epitope (PADRE) associated with several murine and human MHC II. In BALB/c mice, β -gal p876 induced cytotoxic T cells (CTL) and PADRE induced delayed-type hypersensitivity. A solid tumor mass growing in 3-dimensional collagen gel was completely destroyed by anti-p876 CTL when soluble p876 was added. In vivo, however, PADRE in addition to p876 was required to inhibit tumor growth. To test the therapeutic efficacy, BALB/c mice were immunized with AdCMVLacZ and boosted two weeks later with p876 and PADRE in Complete Freund's Adjuvant. Mice rejected 100% D2F2 tumors that were pre-loaded with p876 and PADRE, after they received five injections of the same peptides, every other day, at the tumor sites. Treatment with PADRE alone showed partial protection, but p876 alone had no effect. The anti-tumor activity of PADRE was further tested and ~50% of naive D2F2 tumors growing in PADRE immunized mice were rejected after mice received five local injections of PADRE. Further challenge of D2F2 tumor was again rejected, indicating immunity against endogenous tumor antigens induced by PADRE therapy. Therefore, MHC II peptides may be efficacious therapeutics when delivered to the tumor sites and MHC I peptides can function as therapeutic targets when coated on tumor cells. Department of Defense and NIH CA57831.

**Foreign peptides delivered to the tumor sites are
effective anti-cancer agents¹**

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SUMMARY

T cells are readily activated by immunogenic peptides, whether naturally occurring or artificially designed. A novel immunotherapy strategy taking advantage of peptide specific CD4 and CD8 T cells was tested. Mice were immunized with foreign peptides and the same peptides were delivered to the tumor sites as therapeutic agents. The test agents were L^d associated beta-galactosidase (β -gal) peptide p876 and Pan DR Reactive Epitope (PADRE) presented by several murine and human MHC II. BALB/c cytotoxic T cells (CTL) to β -gal p876 destroyed solid tumor boluses in collagen gel, supporting their anti-tumor activity. BALB/c mice were immunized with p876, injected with p876 loaded tumor cells, followed by injection of p876 at the tumor site. No therapeutic effect was observed. To enhance expansion and survival of CTL in vivo, Pan DR Reactive Epitope (PADRE) which induced delayed-type hypersensitivity in BALB/c mice was tested. Mice were immunized with both p876 and PADRE and injected with tumor cells pre-loaded with the same peptides, followed by five peptide injections at the tumor sites. Complete tumor rejection was achieved. When p876/PADRE immunized mice were challenged with tumor cells not pre-coated with peptides, about 50% rejected tumor after PADRE injection, but p876 had no effect. Vaccination and therapy with PADRE alone also demonstrated anti-tumor effect. Therefore, combined treatment with PADRE and MHC I reactive peptide was highly effective if MHC I peptide was delivered directly unto the tumor cells. Delivery of PADRE alone, but not MHC I peptide, to naked tumors was therapeutically effective. These results indicate foreign peptides as potential therapeutic agents and warrant further development of vehicles for delivering peptides unto tumor cells.

INTRODUCTION

Vaccination with tumor associated immunogenic peptides has been shown to induce anti-tumor immunity. In our lab, the growth of BALB/c mammary tumor was inhibited by vaccination with peptide E474 from the envelop protein of mouse mammary tumor virus (MMTV) (Wei et al. 1996). Vaccination with HLA-A1 restricted MAGE-3 peptide resulted in tumor shrinkage in three of sixteen melanoma patients (Marchand et al. 1995). Immunization with HLA-A2 restricted gp100 peptide combined with systemic IL-2 treatment induced partial response in 42% of melanoma patients (Rosenberg et al. 1998). Patients with metastatic melanoma showed complete response after they were immunized with dendritic cells loaded with three melanoma antigen peptides (Nestle et al. 1998). These examples indicated the anti-tumor efficacy of peptide specific cytotoxic T cells (CTL). Other than melanoma, however, peptide vaccination has demonstrated little efficacy and this may be attributed to the lack of defined tumor associated antigens in other cancer types.

Several limitations in tumor specific immunotherapy prompted the search for alternative approaches in anti-tumor therapy. Antigen expression by the tumor cells is heterogeneous and T cells recognizing tumor specific antigens may eliminate antigen positive tumor cells, but allow the expansion of antigen negative cells. Immunization with multiple antigens may reduce such negative selection, but requires the identification of multiple tumor associated antigens for individual tumors. Many tumor associated antigens are inappropriately or over-expressed self-antigens rather than tumor specific antigens. Separation of auto-immunity and anti-tumor immunity remains a major challenge. In this study, an alternative approach was tested to exploit the effector T cell

function without being limited by the tumor associated antigens. Therefore, mice were immunized with foreign peptides followed by local delivery of these peptides to the tumors as therapeutic agents.

Therapeutic potential of CTL against a solid tumor was tested in vitro in collagen gel (Wei et al. 1985; Wei et al. 1996). Tumor cells grew as a 3-dimensional mass with intracellular junctions and resembled a solid tumor in vivo. The tumor mass could be maintained in the collagen gel for about 2 wks, providing prolonged observation period and a realistic in vitro system to measure anti-tumor activity (Gavin et al. 1993). CTL were co-embedded with the tumor or added exogenously. Activated T cells enter the collagen gel via random motility {Ratner, Patrick, et al. 1992 ID: 1071} and the effect on tumor growth was monitored. The anti-tumor activity of peptide specific CTL was measured in vitro with this realistic system.

Pan-DR Reactive Epitope (PADRE) aK(X)VAAWTLKAAa (a is D-alanine and X is cyclohexylalanine) is a synthetic peptide containing amino acid sequences derived from the circumsporozoite protein (CSP) from Plasmodium falciparum. PADRE has been shown to bind multiple human DR haplotypes and murine I-A^{b/d} or I-E^{b/d} (Alexander et al. 1994) and provide helper T cell activity both in vitro and in vivo. The efficacy of PADRE to enhance anti-tumor CTL activity and as a therapeutic agent alone was examined.

MATERIALS AND METHODS

Mice and cell lines

BALB/c mice originally obtained from the Cancer Research Laboratory, Berkeley, CA were bred by brother-sister mating in our animal care facility. Mouse mammary tumor (MMT) line D2F2 was cloned from a spontaneous mammary tumor which arose in a BALB/c hyperplastic alveolar nodule (HAN) line D2 (Mahoney et al. 1985). MMT line 168 was cloned from a spontaneous tumor in a BALB/cfC3H mouse which was the offspring of BALB/c mice foster nursed on C3H/HeJ mothers infected with MMTV(C3H) (Wei et al. 1993). Line 293 (American Type Culture Collection, Manassas, VA) is a human embryonic kidney cell line transformed with Ad5 E1A and E1B genes and supports the propagation of E1 deleted recombinant adenovirus. All tissue culture reagents were obtained from GIBCO laboratories (Grand Island, NY) unless otherwise specified. The cell lines were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 5% heat inactivated fetal calf serum (HyClone, Logan, UT), 10% NCTC 109 medium (Sigma, St. Louis, MO), 8 µg/ml bovine crystalline insulin (Sigma), 1 mM oxalacetic acid, 0.5 mM sodium pyruvate, 2 mM L-glutamate, 0.1 mM MEM nonessential amino acids, 100 units/ml penicillin and 100 µg/ml streptomycin.

Peptides

β-gal peptide p876 TPHPARIGL and PADRE aK(X)VAAWTLKAAa (a is D-alanine and X is cyclohexylalanine) were synthesized at Genemed Synthesis, Inc., (South San Francisco, CA).

Establishment of peptide specific CTL

BALB/c mice were injected s.c. at the tail base with 100 µg of p876 emulsified in 0.1 ml of 50% Complete Freund's adjuvant (CFA). At 10 days after the injection, the spleen and lymph nodes were aseptically removed from immunized mice and a single cell suspension was prepared. The freshly isolated cells were cultured with peptide-loaded, irradiated splenocytes in 12 well plates in RPMI 1640 containing 50 µM of 2-mercaptoethanol, 10% fetal calf serum, 5 % mixed leukocyte culture supernatant and 18 IU/ml of recombinant hIL-2 (Cetus corp.). The ratio of stimulators to responders was 2:1.

To prepare the stimulators, autologous splenocytes were incubated with 100 µg/ml of p876 for 2 hrs, exposed to 1,000 rad irradiation from a Cesium irradiator and washed to remove excess peptides before they were added to the culture. The cultured cells were re-stimulated with peptide-loaded splenocytes every 7 – 10 days.

Mixed leukocyte culture was prepared by incubating BALB/c splenocytes with irradiated human peripheral blood leukocytes for 48 hrs in RPMI 1640 with 10% FCS. The ratio of mouse and human cells was 1:1 and the total cell concentration was 5×10^6 cells/ml. .

^{51}Cr release assay

D2F2 tumor target cells were labeled by incubating 10×10^6 cells with 100 µCi $\text{Na}^{51}\text{CrO}_4$ (NEN Research Products, Boston, MA) in 1 ml of complete DME at 37°C for 2 hrs. In some experiments, the target cells were loaded with p876 peptide by adding 100 µg of p876 during the incubation. The unincorporated ^{51}Cr and unbound peptide was

removed by three washes with Hanks balanced salt solution containing 2% calf serum and 2 mM Hepes buffer. Graded numbers of effector cells were mixed with 1×10^5 labeled target cells in 200 μ l of DME in the wells of round bottom microtiter plates. After centrifugation at 200 x g for 1 min, the plate was incubated at 37°C for 4.5 hrs. After the incubation, the plate was centrifuged at 480 x g for 10 min and a 100 μ l aliquot was removed from each well for counting in the gamma counter. The percent lysis was calculated as follows:

$$\% \text{ specific lysis} = 100 \times (\text{cpm}_{\text{test}} - \text{cpm}_{\text{medium}}) / (\text{cpm}_{\text{max}} - \text{cpm}_{\text{medium}})$$

The cpm_{max} was determined by adding 1/6 N HCL to wells containing ^{51}Cr -labeled target cells. Each group contained four replicates.

A three-dimensional collagen gel assay

The growth and lysis of tumor cell boluses in the collagen were previously reported and are briefly described here (Wei et al. 1996). Collagen stock solution was prepared from rat tail tendons. The complete collagen mixture contained 12 parts of cold collagen stock, 2 parts of 7.5 x RPMI 1640, 0.6 parts of 7.5% sodium bicarbonate and 0.5 part of 0.34N NaOH. This mixture stayed in liquid form if kept on ice. Tumor cell boluses were prepared by suspending 1×10^5 tumor cells with or without CTL in 1 μ l of complete collagen mixture and embedded between two layers of collagen gels. The gel was bathed in RPMI 1640 supplemented with 50 μ M of 2-mercaptoethanol, 10% fetal calf serum, 5% mixed leukocyte culture supernatant and 18 IU/ml of recombinant hIL-2 (Cetus corp.). Tumor cells established inter-cellular junction and formed a cell mass during overnight culture (Wei et al. 1985; Wei et al. 1996). In some experiments, the

CTL were added to the top of the collagen and allowed to migrate into the gel. Peptide p876 was added the day after CTL were embedded or added. The plates were incubated at 37°C and tumor growth was measured every other day.

Tumor cell boluses embedded in collagen gel were examined with an inverted microscope equipped with a split image tracing device, using 1X objective. The projected image of the cell bolus was traced and the surface area calculated. The result was expressed as the square root of the measured area which represented the mean diameter of the bolus multiplied by a constant. The difference between the test and control group was determined with one tail Students' T test. There were four replicates in each group.

Foot pad swelling test

BALB/c female mice were immunized by s.c. injection with 20 µg of PADRE and 100 µg of p876 in 50% IFA and challenged two weeks later in the foot pads with either or both peptides in 20 µl of saline. The thickness of the feet was measured with a caliper at 24 and 48 hrs after injection. The significance of the swelling was determined by comparing each test group with the saline control group using Students' T test. There were 7-8 mice in each group.

Lymphocyte proliferation assay

Lymph nodes were removed aseptically from mice and single cell suspension was prepared in RPMI 1640 supplemented with 4% fetal calf serum. Suspended cells were distributed to round bottom wells of 96 well plate and each well received 1.5×10^5 cells. Test peptide at varying concentrations or Concanavalin A (con A) at 1 µg/ml was added to the wells. There were four replicates in each group. Cells were incubated in 5% CO₂

at 37°C for four days. Each well received 1 µCi of ³H-thymidine and the cells were further incubated for 4 hrs before they were harvested with Harvester96 (Tomtec, Hamden, CT) and the incorporated ³H was counted with TRILUX liquid scintillation and luminescence counter (Wallac, Turku, Finland).

Preparation of *AdCMVLacZ*

The construction of *AdCMVLacZ* was previously reported (Acsadi et al. 1994). The recombinant virus was purified through two rounds of plaque purification, propagated in 293 cells, and isolated on a discontinuous cesium chloride gradient. The virus was collected and the salt was removed with a Sephadex column. The quantity of viral particles was estimated by measuring the optical density at 260 nm and the plaque forming unit (PFU) was determined by direct plaque assay with 293 cells.

Tumor growth inhibition assay

Female BALB/c mice at 6 wks of age were immunized by intracutaneous injection with 0.1 ml of saline containing 1×10^{10} PFU of *AdCMVLacZ* and boosted, two weeks later, by s.c. injection at the tail base with 0.1 ml of 50% IFA containing 100 µg of p876 and/or 20 µg of PADRE. At 2 weeks after vaccination, mice were injected s.c. on the flank with 1×10^5 untreated or peptide coated D2F2 cells. Starting the day after tumor cell challenge, mice received subcutaneously, every other day, at the tumor site, 0.1 ml of saline containing 100 µg p876 and/or 40 µg PADRE. A total of five injections were administered and tumor growth was monitored weekly. There were eight mice in each group.

RESULTS

Inhibition of tumor growth by p876 specific CTL in vitro

BALB/c CTL to β -gal peptide p876 was established as described in the materials and methods. After the fifth in vitro stimulation, more than 99% of the cells were CD8+ (not shown). CTL were frozen between the seventh and tenth stimulation and they were readily reactivated. The specificity was measured by the ^{51}Cr release assay. CTL lysed peptide p876 coated BALB/c mouse mammary tumor D2F2 cells, but not untreated D2F2 cells (**Figure 1**). Expression of L^{d} by D2F2 cells was verified by flow cytometry (not shown).

The activity of p876 specific CTL against a solid tumor mass was tested in the 3D collagen gel. A total of 1×10^5 D2F2 tumor cells and 3×10^5 CTL were embedded together in 1 μl of collagen (**Figure 1B**). After overnight incubation when the cell bolus was established, p876 at 5 or 20 $\mu\text{g/ml}$ was added to the culture. D2F2 cells co-embedded with anti-p876 CTL, but without exogenous peptide grew progressively. The addition of p876 resulted in complete inhibition of tumor growth, supporting anti-tumor activity of p876 specific CTL.

To test whether CTL can migrate through the matrix to destroy the target tumor, D2F2 tumor cells were embedded in the collagen, incubated overnight and CTL were added on top of the gel (**Figure 1C**). Approximately 20-40% of CTL migrated through the collagen in 18 hrs (Ratner et al. 1992) and floating T cells were removed. Peptide p876 at 5 or 20 $\mu\text{g/ml}$ was added and complete disintegration of the tumor boluses was observed after 5 days of culture. The destruction of tumor cell boluses by migratory anti-

p876 CTL was verified with an independent mouse mammary tumor line 168 which also expressed L^d (**Figure 1D**), supporting anti-tumor activity of CTL to a foreign peptide.

Effect of p876 on tumor growth in BALB/c mice

To test if anti-p876 CTL can inflict the same anti-tumor activity in vivo, BALB/c mice were immunized with *AdCMVLacZ* to activate β -gal specific CTL and boosted in two weeks with 100 μ g p876 in IFA . At two weeks after the second immunization, mice were challenged with D2F2 cells pre-coated with p876. Starting the day after tumor cell challenge, mice were injected at the tumor site, every other day, with 200 μ g of p876 in 0.1 ml saline. There were five injections in 10 days. Control mice received saline or were not treated. One group received uncoated D2F2 cells. There was no demonstrable protection in any of the treatment group (**Figure 2**). The inability to inhibit tumor growth by local administration of p876 may be due to (1) inadequate number of CTL at the tumor site, (2) inadequate cytokine or other signals to sustain CTL activity at the tumor site, or (3) destruction of normal rather than tumor cells marked by the injected peptide.

T cell activation by PADRE

To enhance local recruitment or maintenance of CTL, CD4 T cell reactive peptide was incorporated into the treatment. PADRE which binds to a wide range of human and murine MHC II was tested as local CD4 T cell activator. The immunogenicity of PADRE was tested by the foot pad swelling assay. BALB/c mice were immunized s.c. with 20 μ g of PADRE in 0.1 ml of 50% CFA and challenged two weeks later in the foot pad with 40 μ g of PADRE in 20 μ l of saline. Significant swelling was induced by

PADRE in specifically immunized mice both 1 and 2 days after challenge (**Table I**). Injection of PADRE in control mice or saline in PADRE immunized mice did not induce significant swelling. Therefore, specific T cells were activated in vivo by PADRE immunization. Delayed hypersensitivity induced by a combination of p876 and PADRE was also tested. Mice were immunized with 100 µg of p876 and 20 µg of PADRE in Incomplete Freund's adjuvant and challenged two weeks later with p876, PADRE or a mixture of both peptides. Significant increase in foot pad thickness was observed after PADRE was injected (**Table II**). Injection of both p876 and PADRE further enhanced the immune reactivity. p876 alone did not have significant effect. These results further support the immunogenicity of PADRE in BALB/c mice and an enhanced inflammatory reaction when both MHC I and II reactive peptides were present.

Activation of T cells by PADRE was measured in vitro by lymphocyte proliferation assay (Table 3). Lymph node cells were prepared from BALB/c mice two wk after mice were immunized s.c. with 20 µg of PADRE in 0.1 ml of 50% CFA. Immune cells were incubated with 0.1 to 10 µg of PADRE and ³H-thymidine incorporation was measured after 4 days of culture. Incorporation of ³H-thymidine was increased by more than 100 fold in sensitized lymphocytes when incubated with 10 µg of PADRE. Naïve lymphocytes did not demonstrate significant activity, supporting specific immune activation by PADRE.

Therapeutic effect of p876 and PADRE

To test the therapeutic effect of PADRE and p876, BALB/c mice were immunized with *AdCMVLacZ* and boosted in 2 wks with 100 µg p876 and 20 µg PADRE in IFA. At two weeks after the second immunization, mice received s.c. D2F2 cells pre-coated with p876 and mixed with or without PADRE. Mice were treated five times at the tumor site with p876 alone or a mixture of PADRE and p876, every other day. Complete

inhibition of D2F2 tumor growth was observed in mice treated with PADRE and p876 (**Figure 3**). These results suggested that PADRE activated CD4 T cells at the tumor site enhanced p876 mediated CTL activity and resulted in tumor rejection.

We further tested the efficacy of treating D2F2 tumors which were not pre-loaded with exogenous peptides. Mice were immunized i.c. with AdCMVlacZ and boosted in two weeks with p876 and PADRE. Mice received untreated D2F2 cells two weeks later and peptide injection at the tumor site starting 2 days after tumor cell injection. **Figure 4** shows tumor rejection in 5 of 8 mice treated with PADRE and 4 of 8 mice treated with a mixture of p876 and PADRE. P876 alone had no therapeutic effect. Local treatment with MHC II binding peptide alone appeared to be therapeutic to D2F2 tumor cells not expressing class II MHC.

To test the therapeutic activity of PADRE alone, mice were immunized with 20 µg of PADRE in CFA and boosted with the same peptide in IFA. Injection of D2F2 tumor cells and PADRE peptide at the tumor site were performed as before. Again, half of PADRE treated mice rejected the tumor (**Figure 5**). Mice in the control group were mock immunized with CFA and IFA and injected five times with PADRE at the tumor site. There was no therapeutic effect with this treatment. Therefore, anti-tumor activity of locally delivered PADRE was mediated by previously immunized effector cells.

DISCUSSION

The results from this study indicated therapeutic effect of foreign peptides delivered to the tumors. The anti-tumor activity of peptide specific CTL was profound when tested in the 3-D collagen gel such that a growing tumor was destroyed by anti-

p876 CTL and exogenously added p876. In vivo, tumor cells were completely rejected if they were pre-loaded with p876 and PADRE and treated with the same peptides. Direct loading of p876 onto MHC I of the tumor cells was necessary for CTL to exert anti-tumor activity because D2F2 tumors without peptide pre-loading were not rejected by p876. Treatment of immunized mice with PADRE alone, which induced foot pad swelling and lymphocyte proliferation, was therapeutically effective. This is the first demonstration that foreign peptides can function as anti-cancer therapeutics and warrants further investigation of this new therapeutic strategy. Pre-loading of MHC I peptide like p876 to human tumor in a clinical setting is not possible. There are, however, many unique adhesion molecules, receptors and enzymes in or around the tumor. With properly designed vehicles which target these molecules, specific delivery of MHC I peptides to the tumor cells for in vivo loading will be possible. The efficacy of MHC I associated peptides may be further enhanced by substituting the terminal amino acids with D-a.a. to inhibit protease degradation and enhance the half-life of peptides in vivo. Delivery of PADRE to the metastatic lesions can be enhanced by similar vehicles also.

β -Gal encoded by E.coli LacZ gene is widely used as a genetic tracer. It is also immunogenic and L^d restricted epitope p876 TPHPARIGL was previously defined (Gavin et al. 1993) (Rammensee et al. 1989). Immunization of BALB/c mice with p876 inhibited the growth of a colon carcinoma genetically engineered to express β -Gal, supporting the efficacy of anti- β -Gal CTL (Specht et al. 1997). In our study, p876 was an effective therapeutic agent if the peptide was targeted directly to the tumor cells and delivery strategy toward this goal is underway.

Tumor growth in the 3-D collagen gel is a useful indicator of CTL anti-tumor activity. When CTL were co-embedded with the tumor cells, tumor growth was inhibited although some tumor cells remained after two weeks. When the same number of CTL were added exogenously, less than half migrated to the tumor mass. These migratory T cells caused complete disintegration of the tumor. At least two mechanisms can account for the differential effect of co-embedded and migratory CTL. When CTL were packed in close proximity in the cell bolus, they may recognize neighboring CTL loaded with p876 and lyse each other. Alternatively, tumor cells may demonstrate immune suppressive activity, e.g. Fas ligand on tumor cells may induce apoptosis of activated T cells. (Whiteside and Rabinowich, 1998; Sarma et al. 1992; Zeytun et al. 1997). These mechanisms can significantly reduce the number of functional effectors in the tumor mass. T cells present in human tumors may be similarly ineffective because of the hostile microenvironment. When the hosts are immunized with foreign peptides, peptide specific T cells will reside in the peripheral lymphoid organs and remain functional until they are recruited to the tumor site by the injected peptides. When recruited to the tumor from the periphery, they may be more efficacious than resident T cells against the tumor.

The in vivo therapeutic effect was striking when both CD4 and CD8 reactive peptides were delivered to the tumor which were pre-loaded with peptides. Activation of CD4 T cells appears critical since delivery of p876 alone had no effect. PADRE can bind to I-A^d or I-E^d expressed by the professional antigen presenting cells (APC) or non-professional APC like the endothelial cells (Rose, 1997) and activate CD4 T cells. Interaction between activated CD4 T cells and APC can further enhance the antigen presenting capacity of the latter (Bennett et al. 1998; Schoenberger et al. 1998).

Cytokines generated by activated CD4 are expected to recruit and activate additional APC, CD8 T cells and non-specific inflammatory cells. The activities of all effector cells may contribute to tumor rejection.

Of particular interest and importance is that PADRE treatment alone resulted in tumor rejection. It is noted that the site of peptide injection did not show visible inflammation. PADRE reacts with a broad range of human and murine MHC II and is an attractive agent in clinical application. The unnatural D- rather than L-amino acid at the N- and C-termini modified the disposition of the R groups, rendering the peptide resistant to proteases and prolongs its in vivo half life (Bobde et al. 1994). The unnatural amino acid cyclohexylalanine at position 3 has a rigid conformation which would direct and restrict the conformation of the peptide backbone (Gierasch et al. 1985; Aubrey et al. 1985). These structural properties of PADRE can prolong the half-life of the peptide and enhance its in vivo therapeutic effect. Increased rigidity of the peptide also reduces interaction with non-specific receptors which may occur with more flexible peptides (Giannis and Kolter, 1993). A single immunization with PADRE was sufficient to induce delayed type hypersensitivity in BALB/c mice even though I-A^d was a relatively weak binder of PADRE. The in vivo stability and potent immunogenicity of PADRE makes it an excellent candidate for peptide therapy.

The intriguing observation was that immunization and treatment with PADRE alone resulted in significant therapeutic effect. The mechanism of action is not yet clear and further investigation is warranted. PADRE may activate Th1 or Th2 at the tumor site and these CD4 T cells may have direct anti-tumor effect or activate non-specific effector cells like natural killer cells or macrophages to exert anti-tumor activity.

Alternatively, activated CD4 T cells may enhance the activity of antigen presenting cells or endogenous anti-tumor CTLs although mice which rejected D2F2 tumors after PADRE treatment were not immune to a second challenge with the same tumor (not shown). This lack of "epitope spreading" may indicate the poorly immunogenic nature of D2F2 tumor. Tumors which express significant tumor associated antigens may demonstrate "epitope spreading" following peptide therapy. The fact that treatment with foreign peptides is effective against poorly immunogenic tumors supports the potential of this new therapeutic modality in human tumors which are poorly immunogenic in general.

With the proposed strategy, several major obstacles in tumor immunotherapy, namely, tolerance to self antigens, induction of autoimmunity or the lack of tumor specific antigens may be circumvented. Because the peptide is foreign, tolerance to the antigen is not an issue. After the peptide treatment is terminated, there is no concern on lingering immune reactivity to self-antigens. Furthermore, this therapy does not rely on the expression of tumor specific antigens because the therapeutic peptides are administered exogenously. If necessary, several peptides can be administered simultaneously or sequentially to enhance the therapeutic effect. Each round of peptide treatment represents a booster shot and increased frequency of reactive T cells can be expected. If this therapy strategy is applied clinically, either novel peptides or peptides from recall antigens may be used. Therapeutic peptides may be administered as induction therapy before conventional treatment or as an adjuvant therapy to eliminate residual tumors.

In summary, excellent therapeutic effect was achieved by injection of foreign peptides at the tumor sites. MHC II associated PADRE alone demonstrated therapeutic effect against naïve tumor cells. To enhance the activity of therapeutic peptide, strategies will be developed for prolonged local release or systemic delivery of therapeutic peptides. These studies can lead to a new class of therapeutic agents for cancer immunotherapy.

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FIGURE LEGENDS

Figure 1 Anti-tumor activity of anti-p876 CTL. (A) Cytotoxic activity of anti-p876 CTL measured by the ^{51}Cr release assay. The D2F2 cells were labeled with $\text{Na}^{51}\text{CrO}_4$ in the presence (solid circle) or absence (open triangle) of p876 and chromium release was measured after 4 $\frac{1}{2}$ hr incubation with anti-P876 CTL. There were four replicates in each group. (B-D) Inhibition of D2F2 tumor growth by anti-p876 CTL in 3-D collagen gel. (B) A total of 1×10^5 D2F2 cells were co-embedded with 3×10^5 anti-P876 CTL in 1 μl of collagen. After overnight incubation, p876 at 5 (solid square) or 20 (open triangle) $\mu\text{g/ml}$ was added to the culture. Control group (solid circle) did not receive peptide. Alternatively, 1×10^5 D2F2 (C) or 168 (D) cells were embedded alone in 1 μl of collagen and 3×10^5 anti-P876 CTL were added to the collagen gel after a tumor mass is established overnight. CTL migrated through the gel and P876 peptide was added after 24 hrs. Tumor growth was measured by the square root of the growth area. * indicates significant difference between the control and test groups at $p < 0.005$.

Figure 2 D2F2 tumor growth in p876 immunized and treated mice. BALB/c mice were immunized i.c. with 1×10^{10} PFU of *AdCMVLacZ* and boosted with $\beta\text{-gal}$ p876. At two wks after vaccination, each mouse received s.c., 1×10^5 D2F2 cells coated or not-coated with $\beta\text{-gal}$ p876. Mice injected with peptide coated cells received peri-lesional injection of 200 μg p876 in 100 μl of saline every other day, 5 times, starting the day after tumor cell injection. Control mice received saline injection or were not treated.

Figure 3 Inhibition of D2F2 tumor growth by PADRE and p876. BALB/c mice were immunized i.c. with 1×10^{10} PFU of *AdCMVLacZ* and boosted in two wks by s.c. injection with 100 μ g of p876 and 20 μ g of PADRE in 50% IFA. At two wks after vaccination, each mouse received s.c. 1×10^5 D2F2 (A) or D2F2 coated with β -gal p876 and mixed with PADRE (B-D). Mice in group (C) received peri-lesional injection with p876 (200 μ g) and PADRE (40 μ g) in 100 μ l of saline every other day, 5 times, starting the day after tumor cell injection. Control mice (B) were not treated or received saline injections (D). The lower panel showed the average tumor diameter in groups A-D.

Figure 4 Therapeutic effect of PADRE and p876. BALB/c mice were immunized with *AdCMVLacZ*, PADRE and p876 as described in the legend of Figure 3. At two wks after vaccination, each mouse received s.c. 1×10^5 naïve D2F2. Mice were divided into four groups, each receiving Saline, 40 μ g PADRE, 200 μ g p876 and a combination of PADRE and p876 every other day for 10 days. The lower panel showed the average tumor diameter in test and control mice.

Figure 5 Therapeutic effect of PADRE. BALB/c mice were immunized with 20 μ g of PADRE in CFA, boosted in two weeks with the same peptide in IFA. At two weeks after vaccination, each mouse received 1×10^5 naïve D2F2 and five PADRE treatment. Control group were immunized with CFA and IFA without PADRE and were treated with PADRE five times.

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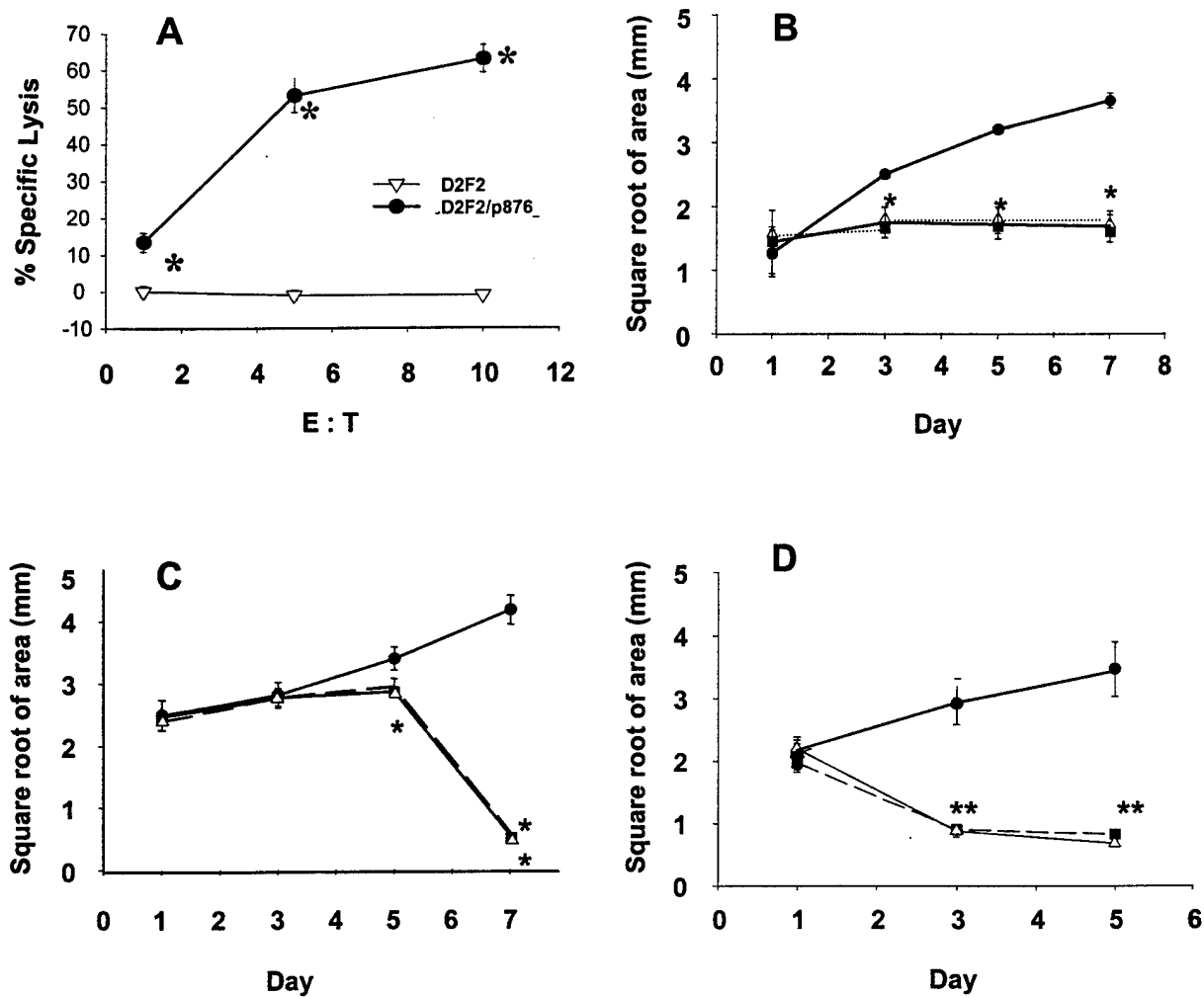


Figure 1

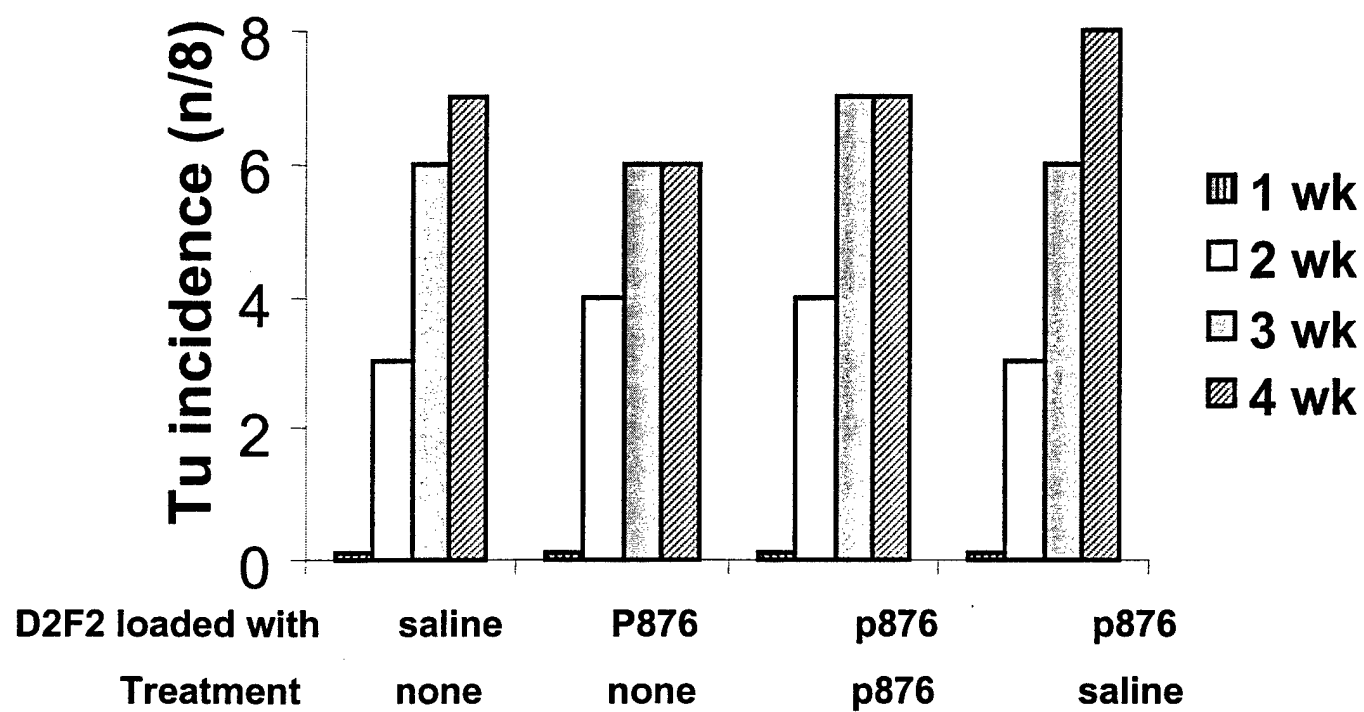


Figure 2

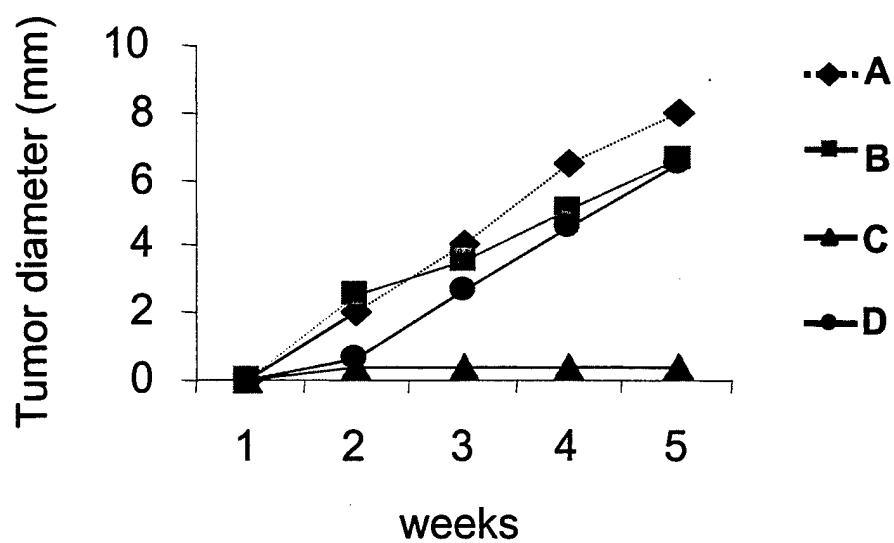
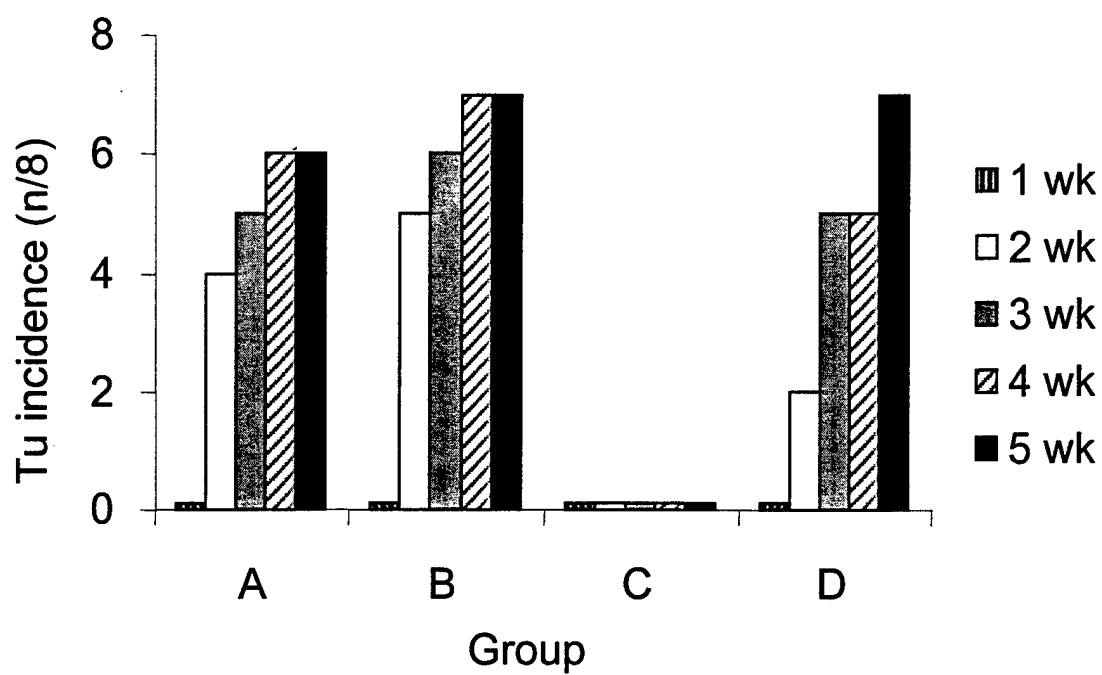


Figure 3

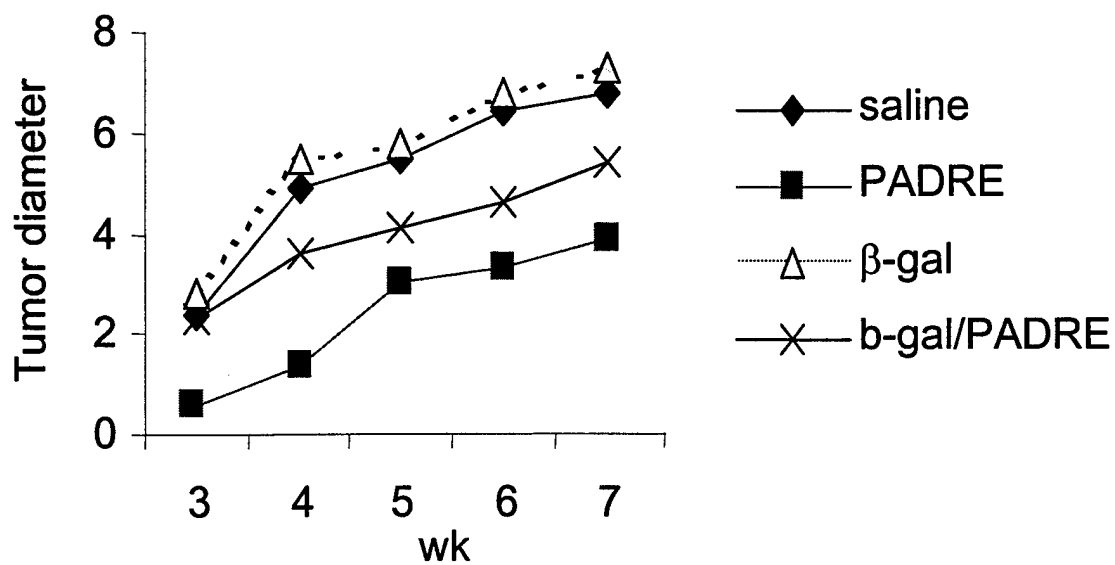
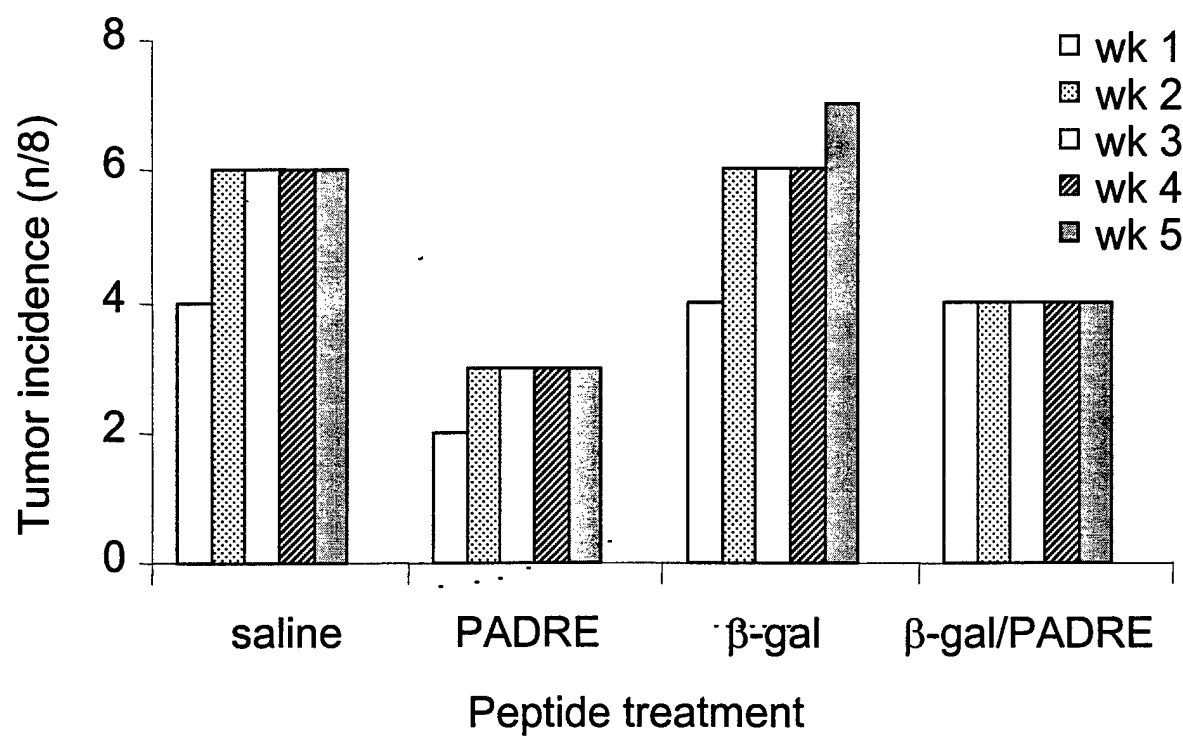


Figure 4

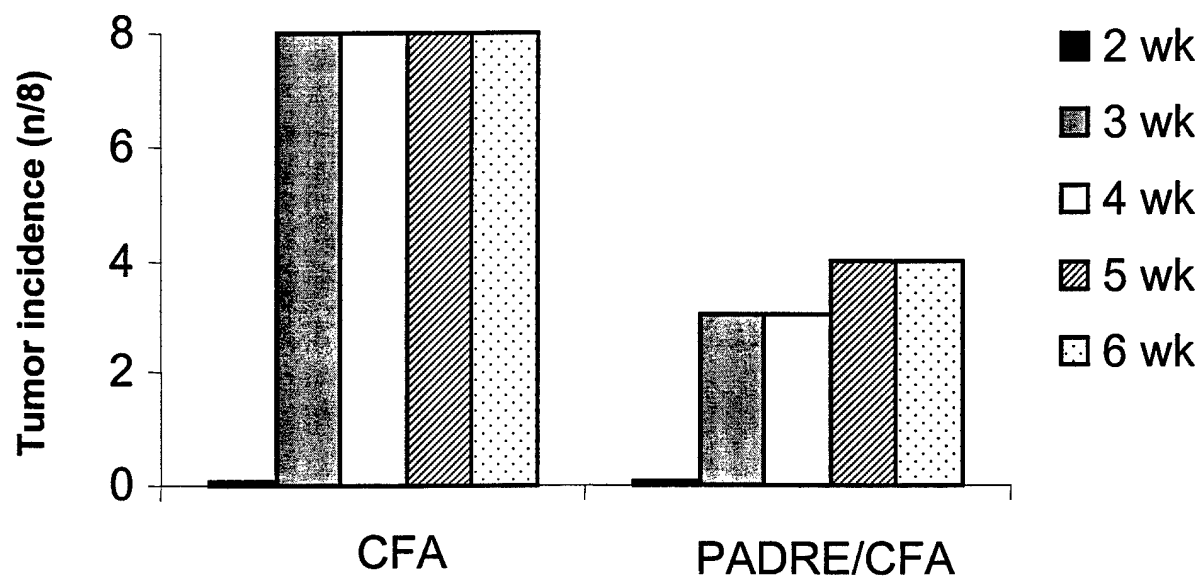


Figure 5

Table 1 Delayed type hypersensitivity induced by PADRE

Vaccination	Challenge	Change in foot pad thickness (mm)			
		Day 1	P value	Day 2	P value*
PADRE/IFA	PADRE	57.4 ± 22.0	<0.0002	36.1 ± 19.3	<0.002
IFA	PADRE	13.4 ± 19.3	>0.05	14.0 ± 13.9	>0.05
PADRE/IFA	Saline	2.9 ± 7.4	>0.05	6.0 ± 18.4	>0.05
IFA	Saline	6.6 ± 16.0	>0.05	3.6 ± 6.6	>0.05

* p value was determined by the student's t test

Table 2 Delayed type hypersensitivity induced by PADRE and β -gal

Challenge	Change in foot pad thickness (mm)			
	Day 1	P value	Day 2	P value*
PADRE	38.6 ± 6.6	<0.001	24.3 ± 8.6	<0.01
β -gal/PADRE	49.3 ± 25.4	<0.05	39.4 ± 18.4	<0.0005
β -gal	12.1 ± 14.7	>0.05	8.7 ± 14.7	>0.05
Saline	3.9 ± 7.1		5.3 ± 9.6	

* p value was determined by the student's t test

Table 3 Lymphocyte proliferation induced by PADRE

Treatment	Immune	mean + S.D. (cpm)		P value*
		P value	Normal	
Medium	145 ± 141		47 ± 14	
PADRE 0.1 μ g/ml	269 ± 136	>0.05	40 ± 10	>0.05
PADRE 5 μ g/ml	6360 ± 974	<0.005	183 ± 117	>0.05
PADRE 10 μ g/ml	18167 ± 3784	<0.005	137 ± 158	>0.05
Con A 1 μ g/ml	92765 ± 11148	<0.005	78263 ± 16138	<0.005

* p value was determined by the student's t test

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